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Synthesis and antimicrobial evaluation of novel 5-substituted-2-(*p*-tert-butylphenyl)benzoxazoles

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In the present study, a series of nine novel 5-substituted-2-(*p*-tert-butylphenyl)benzoxazole derivatives have been synthesized and their structures confirmed by spectral techniques and also tested for their antimicrobial activities. The minimum inhibitory concentrations (MIC) of the new benzoxazoles have been determined against standard bacterial and fungal strains and drug-resistant isolates and compared to those of several reference drugs. The new benzoxazole derivatives are found to possess a broad spectrum of antibacterial activity with MIC values of 8−256 µg/mL. Especially, compound **9** is more active than standard drugs ciprofloxacin and cefotaxime against *E. coli* isolate with a MIC value of 8 µg/mL. Also new compounds are less active than fluconazole with a MIC value of 256 µg/mL against *C. albicans* and its isolate except for compound **9** that shows better activity other compounds with a MIC value of >4 µg/mL for their antifungal activity.

Keywords: Benzoxazole, synthesis, antimicrobial activity

Microbial infectious diseases continue to be one of the leading causes of morbidity and mortality. Especially, the rapidly increasing incidence of multiple drug-resistant Gram-positive and Gram-negative bacteria requires an urgent discovery of novel active agents against these pathogenes[1-3]. Besides, during the past 20 years an increase in invasive fungal infections, particularly in immuno-suppressed patients, has been observed which are now considered to be the causes of morbidity and mortality as well. Thus, new drug classes are urgently needed[4].

Benzoxazoles represent an important class of heterocyclic compounds as structural bioisosteres of nucleotides such as adenine and guanine, which allow them to interact easily with the biopolymers of a living system. Also it was found that benzoxazoles inhibit essential bacterial enzymes. So that, they are useful ligands for more than one type of receptor or enzyme targets by judicious structural modifications for antibacterial and antifungal activity[3-10,16]. A benzoxazole derivative; Routiennocin (Figure 1) that is a spirochetal ionophore antibiotic, isolated from a strain of *Streptomyces chartreusis*. It was found to very active against gram-positive bacteria by acting as a good ionophore[3,14].

Recently, we have described the synthesis of some 2,5-disubstituted-benzoxazoles and their *in vitro* antimicrobial activity against some Gram-positive and Gram-negative bacteria as well as the fungus *Candida albicans*[11-13].

In the present study, a new series of nine novel 5-substituted-2-(*p*-tert-butylphenyl)benzoxazole derivatives**3-11**, has been synthesized using a three-step procedure as shown in Scheme I. In comparison with several control drugs, the newly synthesized compounds were evaluated for their antibacterial and antifungal activity against standard strains and drug-resistant isolates.

Results and Discussion

In the present investigation, a new series of 2-(*p*-tert-butylphenyl-5-substituted- benzoxazoles was synthesized. Their structures were elucidated by mass and ^{1}H NMR spectroscopy and their purity was controlled through elemental analysis. The synthesized compounds were evaluated for their antimicrobial activity in comparison with standard drugs and the results are presented in Table I.

Compounds **3-11** exhibited broad antibacterial activity with MIC values of 128-64 µg/mL against

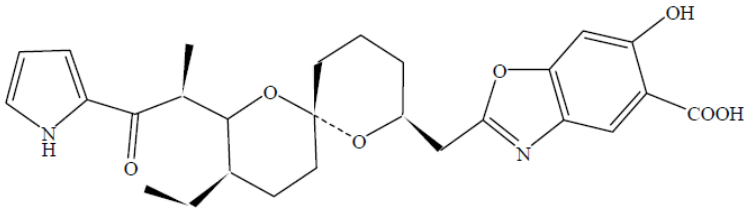
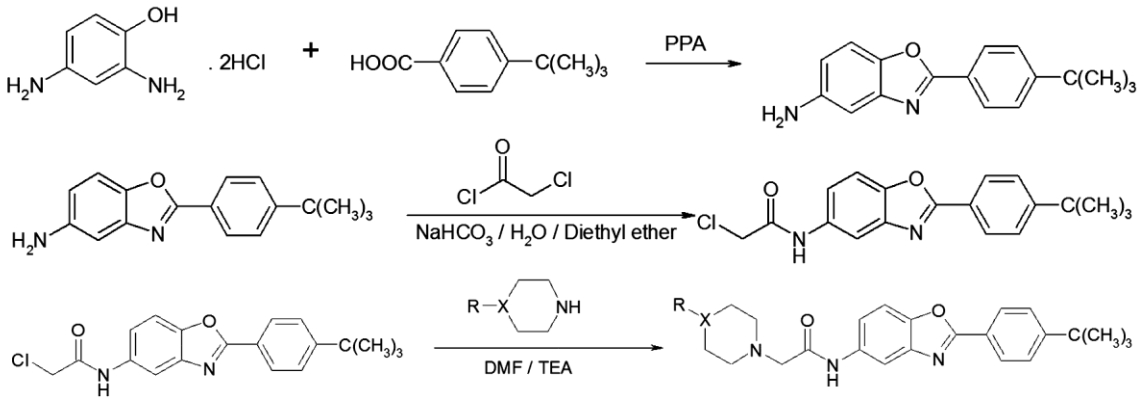


Figure 1 — Routiennocin



Scheme I — Synthesis of the target compounds

Table I — *In vitro* antimicrobial activity of the newly synthesized benzoxazole derivatives in comparison with control drugs (MIC in µg/mL)

			Gram-negative bacteria				Gram-positive bacteria				Fungus	
Compd	X	R	E.c.	E.c.*	P.a.	P.a*	S.a.	S.a.*	E.f.	E.f*	C.a.	C.a.*
3	N	Ø	128	32	128	64	64	128	64	128	256	256
4	N	(*p*-Cl)-Ø	128	32	128	64	64	128	64	128	256	256
5	N	(*p*-OCH_3)-Ø	128	64	128	64	128	128	128	128	256	256
6	CH	Ø	128	128	128	64	128	128	128	128	256	256
7	N	Ø-C=O	64	32	32	64	128	128	64	64	256	256
8	CH	Br	64	64	64	64	128	128	128	128	256	256
9	N	C_2H_5	8	8	8	64	16	16	16	8	?	?
10	CH	H	128	64	64	64	128	128	64	128	256	256
11	N	(*p*-F)-Ø	128	128	64	64	128	128	64	128	256	256
	Ampicillin		4	16	-	-	1	16	0,5	>16	nd	nd
	Ciprofloxacin		0,015	>16	1	8	0,125	>16	0,5	>16	nd	nd
	Meropenem		0,0625	0,0078	0,25	0,25	0,015	-	0,5	nd	nd	nd
	Cefotaxime		0,125	>16	8	>16	2	>16	nd	nd	nd	nd
	Fluconazole		nd	nd	nd	nd	nd	nd	nd	nd	1	1

E.c., *Escherichia coli* ATCC 25922; E.c.*, *Escherichia coli* isolate (ESBL); P.a., *Pseudomonas aeruginosa* ATCC 27853; P.a.*, *Pseudomonas aeruginosa* isolate (Resistant to ciprofloxacin and cefotaxime); S.a., *Staphylococcus aureus* ATCC 29213; S.a.*, *Staphylococcus aureus* isolate (MRSA); E.f., *Enterococcus faecalis* ATCC 29212; E.f.*, *Enterococcus faecium* isolate (VRE); C.a., *Candida albicans* ATCC 10231; C.a*., *Candida albicans* isolate.

nd: not determined (microbiological assays were not performed due to following reasons, antibacterial drugs were not assayed against fungi and antifungal drugs were not assayed against bacteria

S. aureus and the MRSA isolate, except for derivative **9**which had a MIC value of 16 µg/mL. Compound **9** possessed the same or similar MIC values as ampicillin (16 µg/mL) and ciprofloxacin (>16 µg/mL) against its drug-resistant isolate, MRSA. The newly synthesized compounds exhibited antibacterial activities with MIC values between 128-8 µg/mL against *E. faecalis* and its isolate. Among the new compounds, compound **9** was found as the most potent derivative with a MIC value of 8 µg/mL against the vancomycin-resistant isolate of *E. faecalis*, having more potency than the standard drugs ampicillin and ciprofloxacin. All the newly synthesized benzoxazole derivatives exhibited antibacterial activity against the Gram-negative bacteria *E. coli* and *P. aeruginosa* and their respective drug-resistant isolates, with MIC values between 32 and 128 µg/mL, except for derivative **9**, which was the most effective compound against *P. aeruginosa*, *E. coli* and its isolate with a MIC value of 8 µg/mL. Among the newly synthesized benzoxazoles, compound **9** had the same activity as cefotaxime against *P. aeruginosa*.

The tested compounds possessed low antifungal activity against *C. albicans* and its isolate in comparison with a MIC value of 256 µg/mL with the antifungal reference drugs fluconazole except for compound **9** which had a MIC value of >4 µg/mL against these fungi.

Experimental Section

Chemicals and solvents were purchased from Sigma-Aldrich Co. (Taufkirchen, Munich Germany) and Fisher Scientific (Pittsburgh, PA, USA) and used without further purification. Silica gel HF_{254} chromatoplates (0.3 mm) were used for TLC and chloroform was employed as mobile phase. Melting points were recorded on a Stuart Scientific SMP 1 (Bibby Scientific Limited, Stone, Staffordshire, UK) and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury 400 MHz NMR spectrometer (Palo Alto, CA, USA) in $CDCl_3$; tetramethylsilane (TMS) was used as an internal standard. The mass spectra were recorded on a Waters ZQ Micromass LC-MS spectrometer (Milford, MA, USA) using the ESI(+) method. Elemental analyses were performed on an LECO 932 CHNS (St. Joseph, MI, USA) instrument and results were within ±0.4% of theoretical values.

General procedure for the preparation of 5-amino-2-(*p*-tert-butylphenyl)-benzoxazoles, 1

5-Amino-2-(*p*-tert-butyl phenyl)-benzoxazole was synthesized by heating 0.02 mol 2,4-diaminophenol-hydrochloride with 0.02 mol *p*-tert-butyl benzoic acid in 25 g polyphosphoric acid (PPA) and stirring for about 1 h at 160-200°C. At the end of the reaction period, the residue was poured over ice, and the solution was neutralized with 10% NaOH. The resulting precipitate was filtered, washed with distilled water, dissolved in boiling ethanol with 0.2 g charcoal, and filtered off. Crystallization was achieved by dissolving the precipitate in ethanol and adding distilled water. The crude compound **1** was obtained by filtration and drying the filtrate under ambient conditions[13].

General procedure for the preparation of 5-(2-chloroacetamido)-2-(*p*-tert-butylphenyl)benzoxazoles, 2

Chloroacetyl chloride (0.02 mol) was added over a period of 1 h to a stirred, ice-cooled mixture of 5-amino-2-(*p*-tert-butyl phenyl)-benzoxazole derivative (0.02 mol), sodium bicarbonate (0.02 mol), diethyl ether (40 mL), and water (20 mL). The mixture was stirred overnight. The precipitate formed was filtered off, washed with water, and dissolved in ethanol. Crystallization was achieved by adding distilled water, and the crude product **2** was obtained by drying the filtrate under ambient conditions[13].

General procedure for the preparation2-(*p*-tert-butylphenyl)-5-(2-substituted acetamido)benzoxazoles, 3-11

Derivative **2**, 5-(2-chloroacetamido)-2-(*p*-tert-butyl phenyl)-benzoxazole (0.002 mol) was added to 0.002 mol 4-substituted-piperazine/piperidine, 2 mL of triethylamine solution in 3 mL of N,N-dimethyl-formamide (DMF) and 2 mL of ethanol. The mixture was stirred at RT for 24 h. At the end of the reaction time, the mixture was poured over ice, an equal volume of 5% (w/v) of aqueous NaOH solution was added, and the mixture extracted with chloroform. The solvent was evaporated under reduced pressure, and the resulting crude product was purified by column chromatography using chloroform as mobile phase. Finally, the chloroform fractions were collected, the solvent evaporated, and crystallization was achieved by dissolving the residue in chloroform and adding petroleum ether. The crystalline material was dried *in vacuo*. All the compounds **3-11** were prepared as original products. Their structures were supported by spectral data. The ^{1}H NMR, mass spectra and elemental analysis results agree with those of the proposed structures.

2-(*p*-tert-Butylphenyl)-5-[2-[4-phenylpiperazin-1-yl]acetamido]-benzoxazole, 3: Yield 45%. m.p.179°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37 (s, 9H), 2.8-2.9 (t, 4H), 3.25 (s, 2H), 3.3- 3.40 (t, 4H), 6.88 – 6.97 (m, 5H), 7.51 -7.55 (m, 4H), 8.04 (s, H), 8.164 – 8.184 (d, 2H, *J* = 8), 9.27 (s, 1H); LC-MS: *m/z* (%) 469.

2-(*p*-tert-Butylphenyl)-5-[2-[4-[(*p*-chlorophenyl)]-piperazin-1-yl]acetamido]-benzoxazole, 4: Yield 50%. m.p.225°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37 (s, 9H), 2.81 – 2.83 (t, 4H), 3.24 (s, 2H), 3.27 (t, 4H), 6.85-7,25 (m, 4H), 7.51 – 7.55 (m, 4H), 8 (s, H), 8.159 -8.180 (d, 2H, *J* = 8.8), 9.2 (s, 1H); LC-MS: *m/z* (%) 503 (M+H)(100), 505 (33).

2-(*p*-tert-Butylphenyl)-5-[2-[4-[(*p*-methoxyphenyl)]-piperazin-1-yl]acetamido]-benzoxazole, 5: Yield 45%. m.p.201°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35 (s, 9H), 2.82 – 2.85 (t, 4H), 3.17-3,19 (t, 4H), 3.26 (s, 2H), 3.78 (s, 3H), 6.85-6.94 (m, 4H), 7.51-7.55 (m, 4H), 8.04 (s, H), 8.161-8.182 (d, 2H, *J* = 8.8), 9.3 (s, 1H); LC-MS: *m/z* (%) 499 (M+H) (90).

2-(*p*-tert-Butylphenyl)-5-[2-[4-[phenylpiperidine-1-yl]acetamido] benzoxazole, 6: Yield 35%. m.p.173°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.38 (s, 9H), 1.84-2.04 (m, 4H), 2.41-2.61 (m, 3H), 3.078-3.10 (d, 2H, *J* = 8.8),3.21 (s, 2H), 7.22-7.36 (m, 5H), 7.51-7.59 (m,4H), 8 (s, H), 8.169-8.191 (d, 2H, *J* = 8.8), 9.4 (s, H); LC-MS: *m/z* (%) 468.

2-(*p*-tert-Butylphenyl)-5-[2-[4-[benzoylpiperazin-1-yl]acetamido]-benzoxazole, 7: Yield 50%. m.p.184°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37 (s, 9H), 2.68 (m, 4H), 3.58-3.86 (m, 4H) 3.23 (s, 2H), 7.42 (m, 5H), 7.51-7.55 (m, 4H), 7.99 (s, H), 8.156-8.177 (d, 2H, *J* = 8.4), 9.09 (s, H); LC-MS: *m/z* (%) 497 (M+H)(100).

2-(*p*-tert-Butylphenyl)-5-[2-[4-bromopiperidine-1-yl]acetamido]-benzoxazole, 8: Yield 45%. m.p.190°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35 (s, 9H), 2.04-2.11 (m, 2H), 2.24-2.29 (m, 2H), 2.51-2.55 (t, 2H), 2.88-2.93 (m, 2H), 3.18 (s, 2H), 4.25 (s, H), 7.51-7.56 (m, 4H), 8.02 (s, H), 8.16-8.18 (d, 2H, *J* = 8), 9.18 (s, H); LC-MS: *m/z* (%) 470 (M+H)(100).

2-(*p*-tert-Butylphenyl)-5-[2-[4-ethylpiperazin-1-yl]acetamido]-benzoxazole, 9: Yield 40%. m.p.217°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09-1.13 (t, 3H), 1.36 (s, 9H), 2.44-2.56 (q, 4H), 2.7 (m, 6H), 3.17 (s, 2H), 7.49-7.56 (m, 4H), 7.97 (s, H), 8.15-8.17 (d, 2H, *J* = 8), 9.26 (s, H); LC-MS: *m/z* (%) 421 (M+H)(100).

2-(*p*-tert-Butylphenyl)-5-[2-[piperidine-1-yl]acet-amido]-benzoxazole, 10: Yield 30%. m.p.165°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37 (s, 9H), 1.50-1.70 (m, 6H), 2.57 (s, 4H), 3.11 (s, 2H), 7.50-7.57 (m,4H), 8.01 (s, H), 8.16-8.18 (d, 2H, *J* = 8), 9.41 (s, H); LC-MS: *m/z* (%) 392 (M+H)(100).

2-(*p*-tert-Butylphenyl)-5-[2-[4-[(*p*-fluorophenyl)]-piperazin-1-yl]acetamido]-benzoxazole, 11: Yield 25%. m.p.209°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.38 (s, 9H), 2.85 – 2.88 (t, 4H), 3.27 (t, 4H), 3.31 (s, 2H), 6.85-7,25 (m, 4H), 7.61 – 7.64 (m, 4H), 8.04 (s, H), 8.17 − 8.19 (d, 2H, *J* = 8), 9.3 (s, 1H); LC-MS: *m/z* (%) 487 (M+H)(100).

Antimicrobial Activity

Isolates were, *P. aeruginosa* isolate [resistant to ciprofloxacine and cefotaxime], *E. coli* isolate [has Extended Spectrum Beta Lactamase (ESBL) enzyme], *E. faecalis* isolate [resistant to vancomycin (VRE)], and *S. aureus* isolate [resistant to methicilline (MRSA)] and *C. albicans* isolate. Standard strains were, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 10231 standard strains and clinical isolates provided from Trakya University Health Center for Medical Research and Practice (Hospital) were used in the study.

Standard powders of ampicillin (Sigma), cefotaxim (Sigma), ciprofloxacin (Sigma), meropenem (Sigma) and fluconazole (Sigma) were used as standard antimicrobial agents.

Antimicrobial susceptibility testing was performed through CLSI M100-S25 and CLSI M27-A3 directions[15]. Mueller Hinton Agar (MHA) (Merck), Mueller Hinton Broth (MHB) (Merck), Sabouraud Dextrose Agar (SDA) (Merck), Sabouraud Liquid Medium (SLM) (Merck) and RPMI-1640 medium (Sigma) with L-glutamine buffered *p*H 7 with 3-[N-morpholino]-propansulfonic acid (MOPS) (Sigma) were used for microbial cultures.

Stock solutions of the test compounds were prepared in DMSO (Merck). Ampicillin was prepared in phosphate buffer solution and other antibiotic solutions were prepared in sterile distilled water according to the guideline of CLSI M100-S25.

Bacterial isolates were subcultured in Mueller Hinton Agar (MHA) plates and incubated over night at 37°C and *C. albicans* was subcultured in Sabouraud Dextrose Agar (SDA) plates at 35°C for 24-48 h. Pure colonies were transferred to MHB and SLM for bacteria and fungi, respectively. They were incubated in the appropriate conditions overnight. After

incubation, the bacterial suspensions used for inoculation were prepared at 10^5 cfu/mL by diluting fresh cultures at MacFarland 0.5 density (10^8 cfu/mL). Yeast suspensions were also prepared according to McFarland 0.5 density and a working suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension (2.5×10^3 CFU/mL).

Susceptibility testing was performed with MHB for bacteria and RPMI-1640 medium with L-glutamine buffered *p*H 7 with 3-[N-morpholino]-propansulfonic acid (MOPS) for fungi. The solution of the newly synthesized compounds and standard drugs were prepared at 512, 256, 128, 64, 32, 16, 8, 4 µg/mL and 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.0078 µg/mL concentrations, respectively by diluting the stock concentrations in a microdilution tray with a multichannel pipette.

After dilution, a 10 µL bacterial or fungal inoculum was added to each well of the microdilution trays. The trays were incubated at 37°C for bacteria and 35°C for fungi, in a humid chamber and MIC endpoints were read after 24 h of incubation. The lowest concentration of the compound that completely inhibits macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

All organisms were tested in triplicate in each run of the experiments. Solvents, pure microorganisms and pure media were used as control wells. The data on the antimicrobial activity of the compounds and the control drugs as MIC values (µg/mL) are given in Table I.

C**onclusion**

In the recent years we have described the synthesis of some 2-(*p*-tert-butylphenyl)-5-(*p*-substitutedphenyl-acetamido)benzoxazoles as a new class of antimicrobial agents. The derivatives possessed broad *in vitro* antibacterial activity against some Gram-positive and Gram-negative bacteria and their drug-resistant isolates with a MIC value of 128-32 µg/mL. Now we put a *p*-substitutedphenypiperazine/4-ethylpiperazine and 4-substitutedpiperidine-acetamido moiety instead of *p*-substituted phenylacetamido moiety in position 5 of benzoxazole ring. We considered that these groups might improve the antimicrobial activity because position 2 is decisive for the biological activity whereas position 5 is prevailing for the intensity of the activity. 4-(*p*-Substitutedphenyl)piperazyn-1-ilacetamido, 4-substitutedpiperidyn-1-ilacetamido derivatives showed similar activity to *p*-substitutedpheny-acetamido derivatives whereas 4-ethylpiperazyn-1-ylacetamido derivative showed better activity than *p*-substitutedphenylacetamido derivatives.

These observation may serve as a new approach for the design of further antimicrobial agents.

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